Neuroprotective autoimmunity: Naturally occurring $CD4^+CD25^+$ regulatory T cells suppress the ability to withstand injury to the central nervous system

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The ability of rats or mice to withstand the consequences of injury to myelinated axons in the CNS was previously shown to depend on the ability to manifest a T cell-mediated protective immune response, which is amenable to boosting by myelin-specific T cells. Here we show that this ability, assessed by retinal ganglion cell survival after optic nerve injury or locomotor activity after spinal cord contusion, is decreased if the animals were immunized as neonates with myelin proteins (resulting in their nonresponsiveness as adults to myelin proteins) or injected with naturally occurring regulatory $CD4^+CD25^+$ T cells immediately after the injury, and is improved by elimination of these regulatory T cells. In nude BALB/c mice replenished with a splenocyte population lacking $CD4^+CD25^+$ regulatory T cells, significantly more neurons survived after optic nerve injury than in nude mice replenished with a complete splenocyte population or in matched wild-type controls. In contrast, neuronal survival in wild-type BALB/c mice injected with $CD4^+CD25^+$ regulatory T cells immediately after injury was significantly worse than in noninjected controls. These findings suggest that the ability to cope with the sequelae of a CNS insult is affected unfavorably by nonresponsiveness to myelin self-antigens and favorably by conditions allowing rapid expression of an autoimmune response. The regulatory T cells might represent an evolutionary compromise between the need to avoid autoimmune diseases and the need for autoimmunity on alert for the purpose of tissue maintenance.

Injury to myelinated axons in the CNS often has a devastating outcome in terms of neuronal loss, with accompanying loss of function. Part of the damage occurs immediately, whereas part is delayed and leads to the death of neurons that escaped the direct insult. The latter loss is termed secondary degeneration. Autoimmunity has long been viewed as a destructive process. Recent evidence indicates, however, that autoimmunity is the body's endogenous response to CNS injury, and that its purpose is beneficial (1, 2). This notion was based on the observation that in rodents, passive transfer of autoimmune T cells reactive to myelin basic protein (MBP) reduces postinjury neuronal losses relative to those of controls (3–6). It was shown, moreover, that the beneficial effect of such autoreactive T cells is not merely the result of an experimental manipulation, but is a physiological response to a CNS insult (1, 2), because animals devoid of endogenous T cells showed worse outcome of CNS injury than their wild-type counterparts. The ability to exhibit this endogenous autoimmune neuroprotection spontaneously varied among individuals and was, in part, correlated with the individual's genetically determined resistance to the development of an autoimmune disease, such as experimental autoimmune encephalomyelitis (1, 7, 8).

The findings above raised some key questions. Are the T cells that mediate the spontaneous protective mechanism specific to self-antigens? How can we explain the fact that in susceptible animals, which tend to develop an autoimmune disease and therefore are obviously capable of manifesting an autoimmune response, the ability to spontaneously manifest an autoimmune response with a beneficial outcome is significantly weaker than that in resistant strains?

Recent studies have shown that depletion of naturally occurring regulatory $CD4^+CD25^+$ T cells, which comprise ≈10% of the total $CD4^+$ population, predisposes animals to development of organ-specific autoimmune diseases (9). In rats these cells can be depleted by thymectomy at the age of 4 weeks followed by split-dose γ irradiation (10). In mice, thymectomy at the age of 3–5 days prevents the development of regulatory T cells ($CD4^+CD25^+$) but does not alter the repertoire of effector T cells significantly (11).

In this study we provide evidence that the spontaneous protective T cell response to a CNS axonal injury is indeed directed to myelin self-antigens. We also show that depletion of $CD4^+CD25^+$ regulatory T cells enhances the spontaneous T cell-dependent protective response and hence improves postinjury neuronal survival, whereas an increase in the number of regulatory T cells has the opposite effect. Our results suggest that the presence of naturally occurring $CD4^+CD25^+$ regulatory T cells serves to maintain a balance between the ability to manifest an autoimmune response required for neuroprotection and repair and the need to avoid autoimmune disease.

Materials and Methods

Animals. Inbred adult female Lewis and Sprague–Dawley (SPD) rats, adult wild-type BALB/c mice, and nude mice were supplied by the Animal Breeding Center of The Weizmann Institute of Science. Animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee.

Antigens. MBP from guinea pig spinal cord and ovalbumin were purchased from Sigma.

Antibodies and Reagents. Mouse anti-rat CD4 antibodies conjugated to phycoerythrin (PE) and mouse anti-rat CD25 antibodies conjugated to fluorescein isothiocyanate were purchased from Serotec. Rat anti-mouse PE-conjugated CD25 antibody (PC61) was purchased from PharMingen. Mouse recombinant IL-2 and anti-mouse ζ-CD3 were purchased from R & D Systems.

Crush Injury of the Optic Nerve in Rats and Mice. The optic nerve was crushed as described in detail (12). Using a binocular operating microscope, we anesthetized the animals and exposed their right optic nerves. In rats, we used calibrated cross-action forceps to inflict a moderate or severe crush injury on the optic nerve, 1–2 mm from the eye. The severity of the injury determines the number of directly injured neurons. To assess neuroprotection we inflicted a moderate crush injury on the optic nerve in Lewis rats (severe crush in this strain leaves almost no viable retinal ganglion cells (RGCs) because of poor endogenous neuroprotection) and a severe crush

Abbreviations: MBP, myelin basic protein; PE, phycoerythrin; RGC, retinal ganglion cell; SPD, Sprague–Dawley; WSCH, whole spinal cord homogenate; IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant.

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in SPD rats. To assess systemic and local inflammatory effects, we inflicted a severe crush in both strains. Mice were subjected to severe crush injury of the intraorbital portion of one optic nerve. The contralateral nerve was left undisturbed.

Measurement of Secondary Neuronal Degeneration in Rats. Secondary degeneration of optic nerve axons was assessed by retrograde labeling of RGCs, which was done by the application, 2 weeks after crush injury, of the fluorescent lipophilic dye 4-[4-(didecylamino)-styryl]-*N*-methylpyridinium iodide (Molecular Probes) distally to the site of lesion, as described (12).

Retrograde Labeling of RGCs in Mice. The neurotracer dye Fluoro-Gold (5% solution in saline; Fluorochrome, Denver) was injected (3 days before the injury of the optic nerve) into the anesthetized mouse (1 μl, at a rate of 0.5 μl/min in each hemisphere) by using a Hamilton syringe, at a depth of 2 mm from the exposed brain surface, 2.92 mm posterior to the bregma, and 0.5 mm lateral to the midline. One week after crush injury the mice were killed and their retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde solution. Labeled cells from four to six selected fields of identical size (0.7 mm^2) were counted.

Preparation of Splenocytes. Donor splenocytes from rats (aged up to 10 weeks) were obtained by rupturing the spleen and following conventional procedures. The splenocytes were washed with hypotonic buffer (ACK) to lyse red blood cells.

Fluorescence-Activated Cell Sorter Analysis of the α-Chain of IL-2 Receptor (CD25)-Expressing CD4$^+$ T Cells. Cells were immunostained according to manufacturer instructions and were resuspended in 0.4 ml of 1% paraformaldehyde and analyzed by FACSort (Becton Dickinson), with 10,000 events scored. In single-color analysis, positive cells were defined as cells with higher immunofluorescence values, on a logarithmic scale, than those of control cells incubated with isotype antibodies as a control. The cells were scored from a region defined according to physical parameters that indicate the size (forward scatter) and granularity (side scatter) of lymphocytes. CD4$^+$ lymphocytes were then gated for CD25$^+$ analysis.

Depletion of CD25$^+$ Cells. Splenocytes obtained from wild-type BALB/c mice were prepared by the standard procedure and then incubated with rat anti-mouse PE-conjugated CD25 antibody followed by incubation with anti-PE beads (Becton Dickinson). After being washed, the splenocytes were subjected to AutoMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) by using the "deplete sensitive" program. Recovered populations were analyzed by FACSort (Becton Dickinson).

Purification of Murine CD4$^+$CD25$^+$/CD4$^+$CD25$^-$ T Cells. Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleens were harvested and mashed. T cells were purified (enriched by negative selection) on T cell columns (R & D Systems). The enriched T cells were incubated with anti-CD8 microbeads (Miltenyi Biotec), and negatively selected CD4$^+$ T cells were incubated with PE-conjugated anti-CD25 (30 μg/10^8 cells) in PBS/2% FCS. They were then washed and incubated with anti-PE microbeads (Miltenyi Biotec) and subjected to magnetic separation with AutoMACS. The retained cells were eluted from the column as purified CD4$^+$CD25$^+$ cells. The negative fraction consisted of CD4$^+$CD25$^-$ T cells. Cell purity was checked by FACSort and typically ranged from 88% to 95%. Purified cells were cultured in 24-well plates (1 ml) with T cell-depleted spleen cells as accessory cells (irradiated with 3,000 rads) and 0.5 μg/ml anti-CD3, supplemented with 100 units of mrIL-2 (R & D Systems).

Results

Neonatal Immunization of Rats with Myelin Proteins Reduces Their Ability as Adults to Withstand the Consequences of CNS Insult. Our first objective was to determine whether the spontaneous T cell-mediated neuroprotective response to injury of myelinated axons in the CNS is autoimmune in nature and is directed against myelin. It seemed reasonable to assume that if the response is indeed specifically evoked by myelin-associated antigens, eliminating the ability to respond to myelin antigens would diminish the animals' capacity to recover from axonal injury. To test this assumption, we immunized rats at birth with whole spinal cord homogenate (WSCH) emulsified in incomplete Freund's adjuvant (IFA). Previous studies have demonstrated strain-related differences in the spontaneous ability of rats to manifest T cell-mediated protection against the consequences of nerve injuries (7). We therefore first immunized neonates of SPD rats (a strain endowed with the spontaneous ability to manifest a T cell-dependent protective response) with myelin-associated antigens. In adult rats that were immunized with these self proteins as neonates, the number of neurons (expressed as the mean number ± SEM of RGCs per square millimeter) that survived a partial optic nerve crush injury (34.8 ± 1.3) was significantly smaller than in control injured rats that were immunized at birth with ovalbumin (48.4 ± 2.1; $P < 0.01$) or were immunized at birth with IFA without antigen (46.6 ± 3.8; $P < 0.001$; Fig. 1*A*). It is important to note that the number of surviving neurons at any time after injury in this model is a reflection, at least in part, of the balance between injury-induced degenerative processes (12) and the ability of T cell-dependent mechanisms to resist them (2, 6, 7). Since the rats were not subjected to any intervention as adults, meaning that other mechanisms that might affect neuroprotection were untouched, the observed decrease of more than 30% in neuronal survival after CNS injury was purely a reflection of nonresponsiveness to myelin proteins and is therefore of great biological significance. Similarly, when the neonatally immunized rats were subjected as adults to a contusive injury of the spinal cord (3, 13), we found that they had lost the relative advantage normally enjoyed by resistant strains in recovering from a spinal cord injury, because their capacity for spontaneous recovery was completely blocked (Fig. 1*B*). Functional activity after incomplete spinal cord injury is measured by the amount of neural tissue that escaped the primary injury minus the amount of tissue that undergoes delayed degeneration. The latter in turn depends on the rat's ability to withstand the consequences of injury: the more resistant the rat, the better its recovery (7). Our results showed that after spinal cord injury, locomotor activity in an open field was significantly reduced in adult rats that were immunized as neonates with myelin antigens relative to that in rats that were immunized as neonates with ovalbumin with a similar spinal injury. Mean plateau scores (±SEM) of locomotor activity, measured on a scale of 0 (complete paralysis) to 21 (normal mobility) (14), were 3.3 ± 0.4 in the myelin-tolerized rats, reflecting almost complete paralysis, and 6.5 ± 0.8 in the controls, reflecting noncoordinated walking ability ($P < 0.01$). It is important to note that motor activity on this scale is nonlinear, and that the numbers are arbitrary units assigned to different motor skills (14). Therefore, the difference between values of ≈3 and 7 is of great biological significance. Moreover, once the rats reach plateau values the effect is long-lasting (3).

The induced nonresponsiveness to myelin proteins was verified by assessment of the immune response in rats vaccinated with WSCH as adults. Immunization with spinal cord homogenate is known to evoke a strong response to MBP. Therefore, to verify that the neonatal immunization did indeed eliminate the response to myelin-associated antigens, we assayed the ability of the neonatally immunized rats to respond as adults to myelin antigen by immunizing them as adults with WSCH and assaying the ability of their lymphoid organs to proliferate upon challenge with peptides derived from MBP. The proliferative response to MBP after immu-

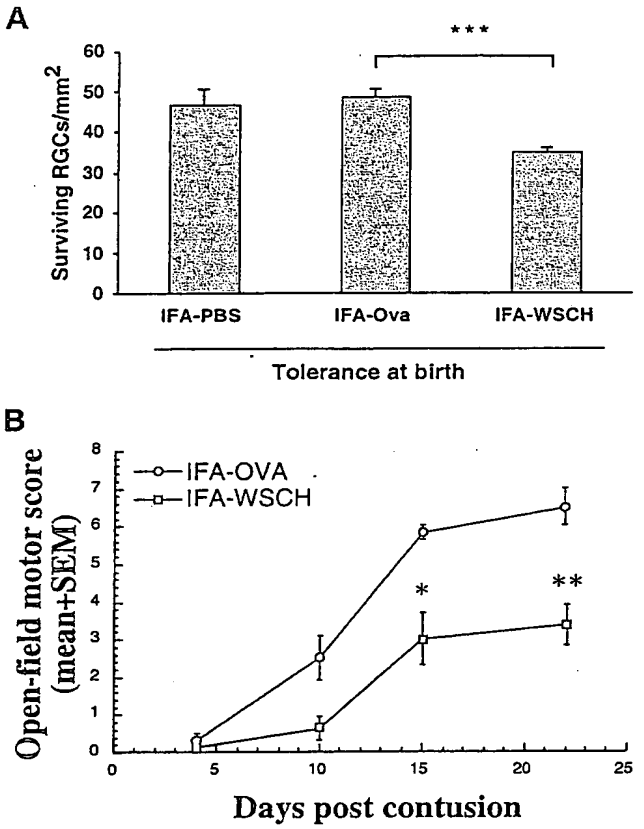

Fig. 1. Neonatal tolerance to myelin antigens abolishes spontaneous neuroprotection after optic nerve crush injury and spinal cord contusion. One-day-old SPD rats were tolerized to myelin antigens by i.p. injection, with 400 μg of WSCH emulsified in IFA. As adults, the rats were subjected to two kinds of CNS injury: (*A*) Partial crush injury of the optic nerve: the numbers of surviving neurons were significantly lower in the rats tolerized to myelin antigens than in control rats that were tolerized 1 day after birth with ovalbumin (OVA) or were not tolerized ($P < 0.01$ and $P < 0.001$, respectively). The figure shows the results of a representative experiment, one of two independent experiments (in each experiment $n = 6-8$ rats in each group). (*B*) Contusive injury of the spinal cord at the level of T9. Recovery was assessed by the locomotor activity score. The locomotor scores of myelin-tolerized contused rats were significantly lower than those of OVA-tolerized rats ($n = 5$ rats in each group; $P < 0.01$; two-tailed Student's *t* test).

nization with WSCH was significantly reduced in the lymphoid organs of rats that were immunized at birth with WSCH compared with control rats that were immunized at birth with ovalbumin (Table 1). We also tested the response to myelin oligodendrocyte glycoprotein in the lymphoid organs of these rats. Because the proliferative response to myelin oligodendrocyte glycoprotein after immunization with WSCH was extremely weak, it was difficult to assess any loss resulting from the neonatal immunization (data not shown).

Lack of a proliferative response in adult rats after neonatal immunization does not necessarily mean that the relevant clones have been deleted. It might be a consequence of an alteration in clonal phenotype (a switch from Th1 to Th2/Th3), resulting in clones that are amenable to activation but only slightly or not at all to proliferation. To verify that the neonatal immunization led to a state of nonresponsiveness and not to a change in phenotype, we compared the activation state of T cells after optic nerve injury in adult rats that had been immunized at birth with WSCH to that in similarly injured rats that had not been immunized as neonates. Activation state was determined by measuring the level of expression of the ergotope IL-2 receptor marker in the lymphoid organs. This marker provides a measure of the expression of the α-chain of the IL-2 receptor $CD25^+$. In naïve rats it measures the amount of regulatory T cells, whereas in activated lymphoid organs (after any challenge) it measures the total amount of activated T cells (whether regulatory or not) and naïve regulatory T cells. In rats that were not immunized with myelin at birth, optic nerve injury at the adult stage resulted in an increase in expression of the CD25 ergotope marker relative to matched uninjured controls (Fig. 2). In contrast, no change in CD25 expression was observed in lymphoid organs of similarly injured rats that were immunized with myelin antigens at birth. Taken together, the results shown in Figs. 1 and 2 suggest that the spontaneous ability to withstand the consequences of CNS axonal insult depends on the ability to activate myelin-specific T cells.

Table 1. Nonresponsiveness of neonatally tolerized rats to challenge by MBP

Tested antigen	[^{3}H]Thymidine, cpm		
	IFA-WSCH* CFA-WSCH†	IFA-PBS* CFA-WSCH†	IFA-WSCH* CFA-OVA†
Ovalbumin	1,133 ± 897	1,381 ± 465	6,584 ± 230
MBP (whole protein)	1,511 ± 577	5,460 ± 541	1,967 ± 138
MBP (peptide 87–99)	1,102 ± 492	10,763 ± 197	1,327 ± 142

Rats were immunized at birth with WSCH and then immunized as adults with WSCH or ovalbumin (OVA) emulsified in complete Freund's adjuvant (CFA) containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra, and their lymph nodes were examined for proliferation by a pulse of ^{3}H-labeled thymidine. After immunization with WSCH in CFA, the neonatally immunized rats (with WSCH) showed a weak proliferative response to MBP or to a dominant epitope of MBP-(87-99). Control rats (neonatally injected with PBS-IFA) responded by strong proliferation of T cells. Neonatally immunized rats (with WSCH) that were immunized as adults with ovalbumin in CFA showed a response similar to that seen in nontolerized controls.
*Neonatal immunization.
†Adult immunization.

Immunization with Myelin Homogenate After CNS Axonal Injury Improves Neuroprotection. As indicated above, the spontaneous ability to manifest a myelin-specific T cell-mediated response is a

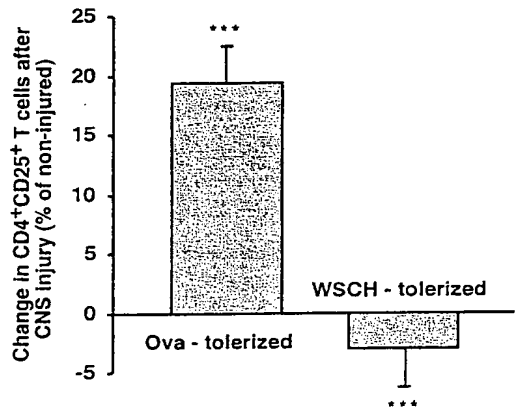

Fig. 2. Nonresponsiveness to CNS injury in rats neonatally immunized with spinal cord homogenate. Three days after being subjected to CNS injury the spleens were removed from rats and verified for T cell activation by using CD25 as an activation marker (ergotope)-α-chain of IL-2 receptor. CNS injury triggered activation of T cells in naïve rats and rats immunized at birth with nonmyelin proteins (Ova-tolerized), whereas activation of T cells was not observed in rats neonatally immunized with myelin proteins (WSCH-tolerized). The results are the mean of three experiments ($n = 3$ in each group in each experiment; $P < 0.001$).

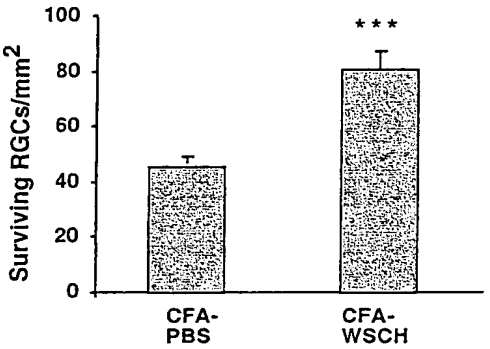

Fig. 3. Immunization of rats with WSCH improves neuronal survival after optic nerve crush injury. Four days before optic nerve injury, Lewis rats were immunized in the hind footpads with 3 mg of WSCH emulsified in CFA supplemented with 5 mg/ml *Mycobacterium tuberculosis* H37Ra. Retrograde labeling of the RGCs showed that significantly more neurons survived in rats immunized with WSCH than in PBS-injected rats. The results shown are of one representative experiment of three independent experiments (in each experiment, $n = 7$–8 in each group; $P < 0.001$, two-tailed Student's t test).

prerequisite for withstanding the consequences of a CNS axonal insult. We considered the possibility that early onset of the T cell response to self-antigens might be the rate-limiting step in developing a beneficial autoimmune response. We therefore attempted to circumvent the limitation in the physiological ability to manifest a beneficial autoimmune response by up-regulating the myelin-specific autoimmune T cells (effector T cells) by means of active immunization with WSCH emulsified in CFA. Active immunization of Lewis rats with WSCH is often done experimentally to induce an autoimmune response culminating in experimental autoimmune encephalomyelitis. This active immunization, although inducing the monophasic disease, led to improved survival of neurons after optic nerve injury (81 ± 7 compared with 48 ± 4; mean number of RGCs ± SEM; $P < 0.001$; Fig. 3).

Depletion of Regulatory T Cells in Rats by Split-Dose Irradiation Improves Spontaneous Neuronal Survival. We considered the possibility that one factor limiting the spontaneous ability to manifest a beneficial autoimmune response might be the constitutive presence of $CD4^+CD25^+$ regulatory T cells. These naturally occurring regulatory T cells, known to be powerful inhibitors of effector T cell activation (10), can be eliminated by split-dose γ irradiation after thymectomy in 4-week-old rats (15). The combined treatment is known to disrupt the regulation of autoreactive clones and increase the frequency of spontaneous autoimmune disease development (9, 15). We found that Lewis rats treated in this way became better able to withstand the consequences of a CNS insult, manifested by an increase of almost 4-fold in neuronal survival after optic nerve crush. The number of surviving neurons per square millimeter in the thymectomized irradiated Lewis rats 2 weeks after optic nerve injury was 113 ± 5 (mean ± SEM), compared with 36 ± 1.5 in control rats that were subjected to crush injury only ($P < 0.001$; Fig. 4*A*). The number of surviving neurons in thymectomized irradiated SPD rats after optic nerve injury was 53 ± 4, compared with 32 ± 3 in SPD rats subjected to crush injury only ($P < 0.01$; Fig. 4*B*).

Depletion of Naturally Occurring Regulatory $CD4^+CD25^+$ T Cells Improves Neuronal Survival After CNS Injury in Mice. The activity of naturally occurring regulatory T cells in mice can be eliminated by thymectomy 3 days after birth (11). We found that significantly more neurons survived severe optic nerve crush injury in adult BALB/c mice that had been subjected to thymectomy 3 days after birth than in nonthymectomized adult mice (737 ± 63 compared

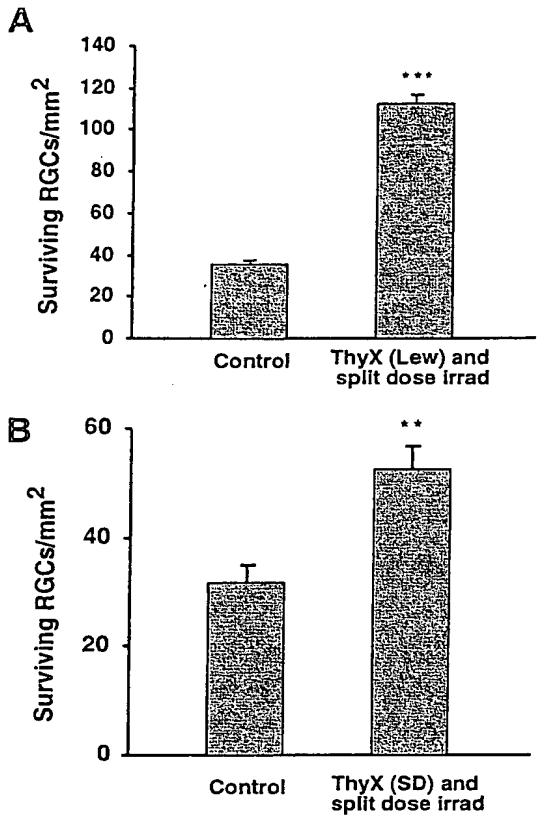

Fig. 4. Thymectomy followed by split-dose irradiation in rats improves neuronal survival. Lewis (*A*) and SPD (*B*) rats (4 weeks old) were thymectomized (ThyX) and then subjected to split-dose irradiation (four bursts of 250 rad each at 2-week intervals). Immediately after the last irradiation the rats were subjected to partial optic nerve crush injury. Neuronal survival was determined 2 weeks later by application of a fluorescent dye. Significantly more neurons survived in the irradiated thymectomized Lewis rats (*A*) than in rats subjected to injury only. The results shown are of one representative experiment of three independent experiments (in each experiment, $n = 7$ in each group; $P < 0.001$, two-tailed Student's t test). (*B*) Significantly more neurons survived in the irradiated thymectomized SPD rats than in SPD rats subjected to injury only ($n = 9$ in each group; $P < 0.01$, two-tailed Student's t test).

with 620 ± 63, $P < 0.001$; Fig. 5*A*). It is important to note that in the mouse optic nerve injury model the total number of surviving RGCs is counted, whereas in the rat optic nerve injury model only RGCs with still-intact fibers are counted (7, 12, 16).

In seeking to provide direct evidence that the regulatory T cells ($CD4^+CD25^+$) are the cells that limit the individual's ability to manifest a beneficial autoimmune response to CNS injury, we examined neuronal survival after optic nerve injury in animals deprived of regulatory T cells. Nude mice on a background of BALB/c were replenished with a normal splenocyte population obtained from wild-type BALB/c mice or with splenocytes deprived of $CD4^+CD25^+$ regulatory T cells. Naturally occurring regulatory T cells comprise ≈10% of the total $CD4^+$ T cell population in naïve spleen. After depletion (see *Materials and Methods*), the percentage of $CD4^+CD25^+$ T cells was <0.5% (data not shown). Before replenishment, the nude mice, as expected (2, 7), showed significantly fewer surviving neurons after optic nerve crush injury than did their wild-type counterparts (Fig. 5*B*). The number of surviving neurons in nude mice replenished with a normal splenocyte population was significantly larger than that in the nonreplenished nude mice (643 ± 27 compared with 412 ± 27; P 0.01), and was slightly (though not significantly) higher than to the

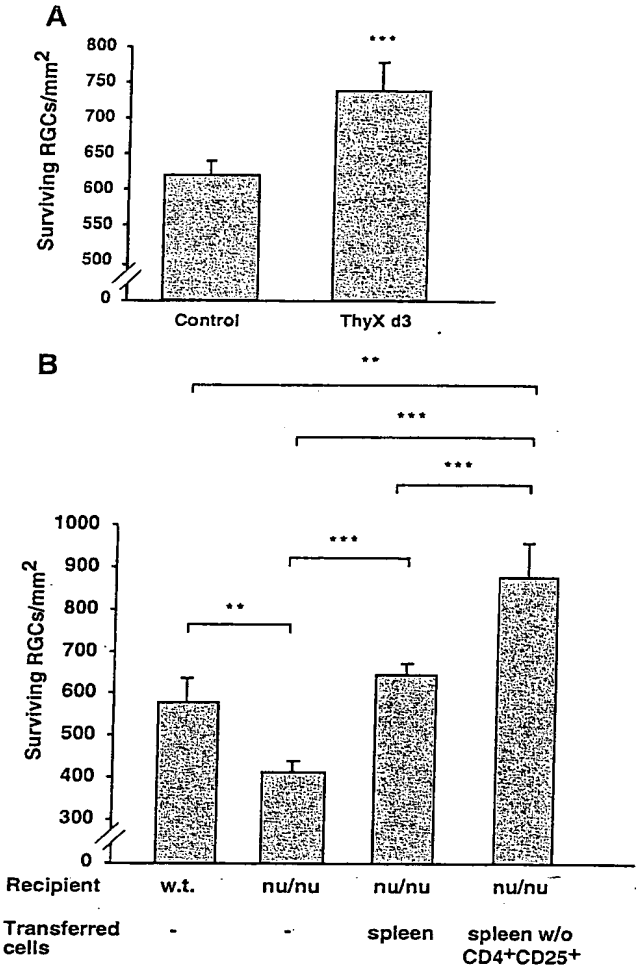

Fig. 5. Depletion of naturally occurring regulatory $CD4^+CD25^+$ T cells in BALB/c mice improves neuronal survival after optic nerve crush injury. (*A*) BALB/c mice were thymectomized (ThyX) 3 days after birth to deplete their regulatory T cells and were subjected as adults to severe unilateral crush injury inflicted on the intraorbital portion of the optic nerve. Surviving neurons were labeled by the application, 3 days before injury, of the neurotracer dye FluoroGold. Significantly more neurons survived in the thymectomized mice than in control (nonthymectomized) age-matched mice ($n = 7–8$ in each group; $P < 0.001$; Student's t test). (*B*) Neuronal survival after optic nerve crush injury in BALB/c *nu/nu* mice (devoid of T cells) was worse than in wild-type mice of matched background. Endogenous neuroprotection in the nude mice was restored by injection of 5×10^7 wild-type splenocytes. Injection of splenocytes depleted of regulatory $CD4^+CD25^+$ T cells increased neuronal survival in these mice beyond even that seen in the wild type ($n = 5–6$ in each group; P values between different groups, obtained by two-tailed Student's t test, are indicated by asterisks above the graph bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

number of surviving neurons found after crush injury in the wild type (577 ± 58). Neuronal survival after optic nerve crush was found to be best in nude mouse recipients of splenocytes lacking $CD4^+CD25^+$ (877 ± 81; $P < 0.001$).

Naturally Occurring Regulatory $CD4^+CD25^+$ T Cells Diminish the Ability to Withstand the Effects of CNS Injury. To examine the direct effect of regulatory T cells on the ability to resist consequences of CNS injury, we measured neuronal survival in wild-type BALB/c (endowed with spontaneous ability to manifest a T cell-dependent protective mechanism) after optic nerve injury and injection of regulatory $CD4^+CD25^+$ T cells. Three groups of BALB/c mice

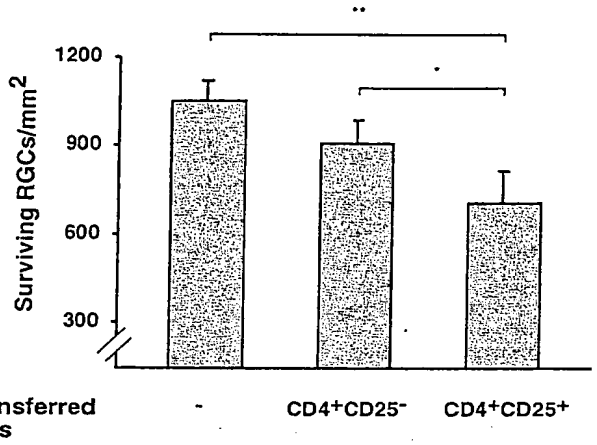

Fig. 6. Naturally occurring regulatory $CD4^+CD25^+$ T cells diminish spontaneous neuroprotection. BALB/c mice, endowed with the spontaneous ability to evoke a protective beneficial autoimmune response, were injected with 2×10^6 purified activated regulatory $CD4^+CD25^+$ T cells. Control mice were injected with 2×10^6 purified activated effector cells ($CD4^+CD25^-$) or with PBS. Injection of regulatory T cells had an adverse effect on neuronal survival (more neurons underwent secondary degeneration). The results shown are of one representative experiment of three independent experiments; $n = 5–6$ mice in each group ($P < 0.01$ and 0.05; respectively).

were subjected to optic nerve crush injury and immediately afterward were injected with activated $CD4^+CD25^+$ regulatory T cells, $CD4^+CD25^-$ effector T cells, or vehicle (PBS). Neuronal survival was determined 2 weeks later. The purity of the isolated $CD4^+CD25^+$ cells ranged between 88% and 95% in different experiments. (Three independent experiments were performed; $n = 5–6$ in each group in each experiment.) Mice injected with the regulatory $CD4^+CD25^+$ T cells showed significantly worse neuronal survival (705 ± 111; $P < 0.01$) than PBS-injected mice ($1,050 \pm 70$) or mice injected with effector ($CD4^+CD25^-$) T cells (906 ± 80) (Fig. 6).

Discussion

The ability of individuals to cope with the consequences of insults to myelinated CNS axons was found in this study to be decreased by neonatal immunization with myelin proteins or by injection (as adults) of naturally occurring regulatory $CD4^+CD25^+$ T cells. As a corollary, postinjury neuronal survival was improved after the rats were immunized as adults with myelin proteins emulsified in adjuvant or after depletion of regulatory (suppressor) $CD4^+CD25^+$ T cells.

Naturally occurring regulatory T cells [apparently the cells that in early studies were identified as thymus-induced suppressor CD4 T cells (17–19)] may be viewed as safeguards against autoimmune disease. At the same time, as suggested by findings during the past few years (3, 5, 7) and as shown in the present work, autoimmune T cells are required as safeguards of CNS maintenance in the day-to-day need to cope with stressful conditions. To reconcile the two apparently opposite, and even conflicting, requirements of avoiding autoimmune disease and manifesting an autoimmune response to avoid neuronal degeneration, a compromise is required. We suggest that such a compromise, in the interest of the well-being of the individual, is achieved, in part, by the presence of the $CD4^+CD25^+$ regulatory T cells, and might provide a mechanism that allows differential activation of some, but not all, of the autoimmune T cells.

The experimental use of protocols known to deplete $CD4^+CD25^+$ T cells was found to lead to an increase in neuronal survival after CNS insults in both susceptible and resistant rat and

mouse strains. This finding suggests that even in individuals capable of spontaneously manifesting an endogenous autoimmune protective response to CNS insults, the response is not of maximal strength, and can be augmented by therapeutic boosting.

To verify that $CD4^+CD25^+$ regulatory T cells are the cells that inhibit the spontaneous ability to achieve maximal activation of autoimmunity, we replenished CNS-injured nude mice on a background of BALB/c with splenocytes deprived of $CD4^+CD25^+$ T cells. Replenishment with these deprived splenocytes resulted in the postinjury survival of significantly more neurons than in wild-type controls. These findings suggest that although an endogenous T cell-mediated neuroprotective mechanism exists in this strain, its expression is limited by the inhibitory presence of the regulatory T cells. Disruption of this regulation can be viewed as a way to increase spontaneous immunization against self, thereby increasing the strength of the beneficial autoimmune response, in some cases, however, at the cost of predisposing the injured animal to the development of autoimmune diseases. Direct evidence for a suppressive effect of $CD4^+CD25^+$ T cells was seen when these cells were passively transferred to BALB/c mice after the injury and fewer neurons survived. Our results thus suggest that nonresponsiveness to myelin self-antigens unfavorably affects the individual's ability to cope with the sequelae of a CNS insult. Neonatal tolerance has been extensively described in the literature, generally in relation to peptides and proteins (20, 21). In this study we demonstrated a loss of responsiveness to MBP, indicated by the proliferation of lymph node cells in response to MBP challenge after neonatal immunization with WSCH. In rats immunized with WSCH emulsified in a strong adjuvant, such as complete Freund's adjuvant, with a high bacterial content, the response is mainly directed to MBP (22). Nonresponsiveness to MBP does not rule out the possibility of a phenotype switch toward a Th2/Th3 response rather than a loss of the response (23–26). However, elevated expression of the ergotope marker after axonal injury, and the loss of such elevation if the rats are immunized at birth with myelin antigens, suggest that neonatal immunization eliminates the ability to activate myelin-specific T cells rather than causing a phenotype switch. Our results therefore suggest that nonresponsiveness of self-reactive clones does not favor adaptation to adverse conditions.

According to our data, if nonresponsiveness is indeed the physiological state of autoimmune clones, this state would negate the very reason for the existence of the immune system, namely the achievement of optimal adaptation. Our results further suggest that the existence in normal individuals of autoreactive clones (or at least those directed against myelin proteins) is not accidental. On a daily basis, these autoimmune T cells are on alert, functioning adequately (and usually below the threshold of the individual's awareness) in maintenance and defense against ubiquitous agents of self-destruction. When a chaotic situation arises, caused for example by traumatic injury, intervention by the autoimmune T cells is urgently needed, but apparently is insufficient, possibly due in part to the presence of $CD4^+CD25^+$, and needs to be boosted to be effective. Boosting can be achieved by up-regulating the effector autoimmune T cells [by active immunization (3, 13, 16)] or by down-regulating the regulatory (suppressor) T cells. Thus, the mechanism that allows an autoimmune disease to be avoided is also the mechanism that limits protective autoimmunity. A compromise, in the form of a balance between these conflicting activities, leads to an autoimmune response which, in terms of day-to-day maintenance, might be sufficient to withstand mild injuries in resistant strains, but is insufficient for extreme conditions such as severe injuries.

It is not only the proportion of regulatory T cells that determines the ability to withstand the consequences of a CNS insult, but also the epitope specificity of the autoimmune T cells in the periphery (the potentially pathogenic autoimmune T cells in susceptible strains), which might in turn also determine the proportion of the regulatory T cells. In any event, both the up-regulation of effector T cells (by immunization) and the down-regulation of regulatory T cells (by irradiation or depletion) lead to an increased ability to recover from a CNS axonal insult.

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